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PRINCIPAL INVESTIGATOR: Christina Soohoo

CONTRACTING ORGANIZATION: Beth Israel Deaconess Medical Center
Boston, Massachusetts 02215

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Christina Soohoo

E-mail: soohoo@fas.harvard.edu

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14. ABSTRACT

Aim 1 is to determine if PinX1 levels are altered in breast cancer tissues and cell lines. We have currently identified several breast cancer samples with reduced PinX1 levels and at least two with barely detectable expression when compared to normal breast tissue. Our screen to identify mutations in the PINX1 gene in breast cancer samples (Aim 2) has thus far identified four C-terminal mutations which are currently being tested for functional significance. To define the region of PinX1 responsible for telomerase inhibition (Aim 3), we have generated a series of truncated proteins and tested their ability to bind to and inhibit telomerase. We have further defined the residues responsible for PinX1 activity in telomerase inhibition and tumorigenesis by generating PinX1 mutant proteins harboring single and multiple amino acid substitutions of conserved residues within the minimal TID and of breast cancer genetic alterations identified in our screen. Our results indicate that the mutation of several conserved residues are sufficient to disrupt hTert binding and telomerase inhibition and that the mutations identified in our screen disrupt telomerase activity slightly and may be crucial in the development of cancer. These results reflect substantial progress toward determining the mechanism and role of PinX1 in breast cancer.

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Breast Cancer, Tumor Suppressor, Telomerase, Telomerase Inhibitors, Telomeres, Cell Growth Control

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INTRODUCTION

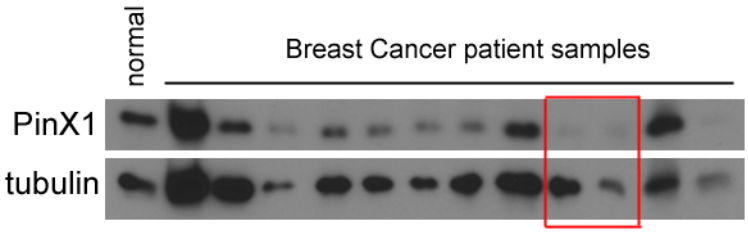
Telomerase activity is critical for normal and transformed human cells to escape from crisis and is implicated in oncogenesis. Our lab has previously identified the novel telomerase inhibitor PinX1 in a yeast two-hybrid screen using Pin2, a telomere-associated protein that negatively regulates telomere length, as bait. Although it has been shown that telomere-binding proteins such as Pin2 regulate telomere maintenance, their mechanism remains to be elucidated since they have not been shown to directly affect telomerase activity. Interestingly, PinX1 inhibits telomerase activity and telomere elongation by binding to the catalytic subunit of telomerase, hTert, with its C-terminal telomerase inhibitory domain (TID). Consequently, depletion of endogenous PinX1 in cells increases telomerase activity and tumorigenicity in nude mice, whereas overexpression of PinX1 or its TID forces cancer cells into growth crisis and eventually senescence. This suggests that PinX1 may be a tumor suppressor, which is supported by the fact that the *PINX1* gene is located on chromosome 8p23, a region with frequent loss of heterozygosity (LOH) in many human cancers.

In order to define the region of PinX1 responsible for telomerase inhibition, we have generated a series of truncated proteins and tested their ability to bind to and inhibit telomerase. We have identified a minimal telomerase inhibition domain (TID) in the C-terminal of PinX1 that is highly conserved among organisms, including yeast in which PinX1 is functionally conserved. Interestingly, several genetic alterations within the extreme C-terminus of PinX1 have been identified in breast cancer tissues and cell lines screened for PinX1 mutations, further emphasizing the significance of this region in oncogenesis. To elucidate the mechanism of telomerase inhibition by PinX1 and its significance in tumorigenesis, we have generated a series of truncations, and single and multiple amino acid substitutions in PinX1 as well as identified a number of genetic alterations in the PINX1 gene in human cancer tissues and cell lines. Specifically, we want to understand whether and how genetic alterations affect PinX1 function and identify residues important for telomerase inhibition and binding. These mutant proteins were expressed and purified to examine how they affect the ability of PinX1 inhibit telomerase and bind to hTERT or Pin2. Therefore, understanding PinX1 function may reveal the mechanism of telomerase regulation at telomere ends.

BODY

Task 1. To determine if PinX1 expression is altered in breast cancer, Months 1-12.

Status. Immunoblotting of human breast cancer tissues has currently identified several samples with reduced PinX1 levels and at least two with barely detectable expression when compared to normal breast tissue. Tissue sections corresponding to the samples with low PinX1 levels will be immunostained to determine if the low levels influence PinX1 cellular localization or cell morphology. We will continue to acquire and analyze additional breast cancer tissues as well as cell lines.



Task 2. To screen for PINX1 genetic alterations in breast cancer tissues and cell lines, Months 6-24.

Status. We have currently identified four amino acid changes of interest in the *PINX1* gene in breast cancer tissues located adjacent to the minimal catalytic domain of PinX1. We are continuing our screen to determine if a pattern of clustering of genetic alterations exists in this region, which would emphasize the importance of this domain.

Task 3. To understand how genetic alterations affect PinX1 function and to identify the residues in PinX1 important for telomerase inhibition, Months 12-36.

Status.

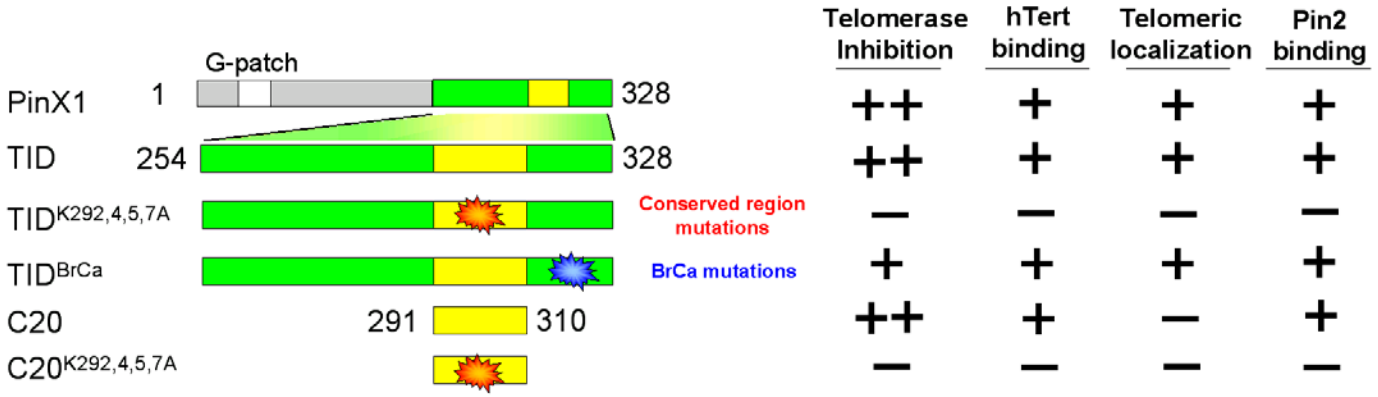
a. We have introduced the genetic alterations identified in human breast cancer samples into PinX1 cDNA and purified exogenously expressed mutant proteins. Thus far, no significant effect on hTert and Pin2 binding or telomeric localization has been determined from these mutations. However, the combined effect of three breast cancer alterations resulted in reduced telomerase inhibitory activity in TRAP assays. Combining the mutations did not affect hTert or Pin2 binding. Therefore, the genetic alterations we identified in our screen:

    1. disrupt telomerase inhibition activity slightly
    2. do not affect hTert binding

3. do not affect Pin2 binding
4. do not affect telomeric localization

b. We have purified a series of PinX1 truncations in order to map a minimal domain with telomerase inhibitory activity. These truncated proteins have defined a 20-amino-acid C-terminal region with activity comparable to the full-length protein. Within this 20-amino-acid region we have identified several highly conserved residues and targeted them for mutation. Although single mutations have not revealed any one critical residue, multiple mutations have identified four residues that in conjunction abolish PinX1 activity. Specifically, mutation of residues K292, K294, K295 and K297 to Alanine:

1. disrupts telomerase inhibitory activity in telomeric repeat amplification (TRAP) assays.
2. ablates binding with telomerase catalytic subunit hTert in GST-pulldown assays using GST-PinX1 mutant proteins and either in vitro transcribed hTert or transiently expressed HA-hTERT.
3. abolishes binding with Pin2, a telomeric protein used as bait to identify PinX1 in a yeast-two-hybrid screen, in GST-pulldown assays
4. eliminates telomeric localization of PinX1 likely through its impaired binding to the Pin2 telomere-associated protein.



Future Aims:

1. Stably express PinX1 mutants lacking telomerase inhibitory activity in telomerase-positive and negative cell lines to assay in vivo role in telomerase regulation.
   a. Test telomerase activity in these cells (TRAP assays).
   b. Determine telomere lengths by Southern blot analysis of telomere restriction fragments (TRF) lengths.
   c. Assay cell growth, morphology and proliferation of cells by BrdU incorporation and flow cytometry.
   d. Assay ability of stable cell lines to initiate tumor formation when injected subcutaneously into nude mice.

KEY RESEARCH ACCOMPLISHMENTS

- Identified several breast cancer tissue samples with reduced PinX1 protein levels and at least two samples with almost undetectable levels.

- Located four genetic alterations in the *PINX1* gene in breast cancer samples clustered adjacent to the minimal domain required for PinX1 telomerase inhibitory activity.

- Purified truncated PinX1 proteins harboring the breast cancer mutations in combination showed reduced telomerase inhibition, but maintained hTert binding, Pin2 binding and telomere localization.

- Defined a 20-amino-acid minimal domain at the C-terminus of PinX1 that is necessary for PinX1 telomerase inhibition.

- Mutation of several key residues conserved among species within the 20-amino acid domain disrupts PinX1 telomerase inhibition, hTert binding, Pin2 binding and telomere localization.

REPORTABLE OUTCOMES

- Oral Presentation and Poster Presentation– Experimental Biology 2006 Conference, Moscone Center, San Francisco, CA. April 1-5, 2006. Telomeres and Aging Session.

- Graduate Student Travel Award Recipient – Experimental Biology 2006 Conference.

CONCLUSIONS

We have made considerable progress toward evaluating the tasks outlined in our original proposal to determine the role of PinX1 in breast cancer and tumorigenesis. Thus far we have successfully identified a region of PinX1 crucial for its role in telomerase inhibition and oncogenesis. Although the breast cancer mutations identified in our screen have had only subtle effects on PinX1 activity, we will continue *in vivo* assays of these mutant proteins. It may be possible that our *in vitro* assays lack the sensitivity required to determine if the reduced telomerase activity is sufficient to have dramatic effects on cell growth and proliferation. Furthermore, we intend to continue screening breast cancer tissue samples and cell lines for reduced PinX1 protein and mRNA levels as well as genetic alterations targeted around the minimal telomerase inhibitory domain. This will further support a significant role for PinX1 in breast cancer as a tumor suppressor.